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Remineralization of demineralized dentin using a dual-analog system

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Abstract

Objectives Improved methods are needed to remineralize dentin caries in order to promote conservation of dentin tissued and minimize the surgical interventions that are currently required for clinical treatment. Here we test the hypothesis that bulk substrates can be effectively mineralized via a dual-analog system proposed by others, using a tripolyphosphate “templating analog” and a poly(acrylic acid) or poly(aspartic acid) “sequestration analog”, the latter of which generates the polymer-induced liquid-precursor (PILP) mineralization process studied in our lab.

Material & Methods – Demineralized human dentin slices were remineralized with and without pre-treatment with tripolyphosphate. Both poly(acrylic) acid and poly(aspartic acid) were used as PILP process-directing agents or “sequestration analogs”. A control experiment with no polymer present was also conducted.

Results – No mineralization was observed in any of the poly(acrylic acid) groups. In both the poly(aspartic acid) and no polymer groups, tripolyphosphate inhibited mineralization on the surfaces of the specimens and promoted mineralization within the interiors of the specimens. Pre-treatment with tripolyphosphate enhanced overall mineralization of the poly(aspartic acid) group. However, when analyzed via TEM, regions with little mineral were still present.

Conclusion – Poly(acrylic acid) was unable to remineralize demineralized dentin slices, even when pre-treated with tripolyphosphate. However, pre-treatment with tripolyphosphate enhanced overall mineralization of specimens remineralized using poly(aspartic acid).

Keywords: Caries; Collagen; Dentin; Mineralization; PILP

1. Introduction

Due to the short longevity of many dental restorations, researchers are exploring biomimetic methods to restore dentin lesions to their natural state. We discovered a way to mineralize collagen fibrils with intrafibrillar hydroxyapatite (HAp). . We term the process the polymer-induced liquid-precursor (PILP) process. PILP uses anionic polypeptides (*e.g.* polyaspartic acid, pAsp) in solution supersaturated with calcium and phosphate ions to sequester ions to induce/stabilize nanodroplets of liquid-like, amorphous calcium phosphate (ACP) precursor phase. These nanodroplets infiltrate collagen fibrils, perhaps by capillary action or the Gibbs-Donnan effect.[1-2] After infiltration, the precursor solidifies into ACP and finally crystallizes into HAp, resulting in large amounts of aligned, intrafibrillar mineral.[1, 3-4] When the PILP process was used to remineralize artificial dentin caries, full mineral density recovery was obtained. However, nanoindentation revealed that the modulus was not fully restored near the surfaces of the lesions, resulting in values ~50% of those of native dentin.[5] We hypothesize that the mechanical properties were not restored due to preferential infiltration of PILP droplets into the interiors of collagen fibrils, resulting in small, intrafibrillar crystals. In native dentin, larger, interfibrillar crystals are also present and are believed to augment mechanical properties.[6-7]

Tay and colleague's research group also performed work with collagen mineralization, both on individual fibrils and within dentin artificial lesions.[8-12] Their system using poly(acrylic acid) (PAA), with and without collagen pre-

treatment with tripolyphosphate (TPP), has shown promise. This method that combines TPP pre-treatment with subsequent mineralization in the presence of PAA is called a dual-analog system, which they believe mimics the roles of non-collagenous proteins (NCPs) responsible for biological mineralization. TPP is termed the “templating analog”, which mimics the highly phosphorylated domains within many of these NCPs. The phosphorylated groups are believed to bind to/interact with the amine groups on collagen. PAA is considered the “sequestration analog” because it contains repeated carboxylic acid side chains that mimic the acidic domains on many NCPs which are thought to play the important role in stabilizing ACP precursors. They argue that although the sequestration analog alone can lead to high amounts of intrafibrillar mineral, the mineral is not “hierarchically”organized as is frequently seen in native dentin.[10]

This paper conducts experiments on demineralized dentin slices, borrowing conditions developed by Tay *et al.,* in the hopes of optimizing our current mineralization methods. We hypothesized that their reported “hierarchical mineralization” might lead to a more uniform penetration depth into the dentin by first creating the higher amount of mineral in the gap zones before it then spreads and forms an interfibrillar mineral cement [13]. TPP pre-treatment was explored in conjunction with mineralization using either PAA or pAsp as the process-directing agent (*i.e.*, “sequestration analog”). The only group that showed promise was TPP pre-treatment and subsequent remineralization using pAsp. However, neither PILP alone nor

PILP with TPP pre-treatment resulted in full remineralization throughout the substrates.

2. Material and Methods

2.1 Sample Preparation

Human third molars were sliced parallel to the occlusal surfaces using an IsoMet wet, circular saw (Buehler, Lake Bluff, IL) with a diamond blade. Slices ~300 µm thick were demineralized in 1 L of 0.5 M EDTA in 0.5 M Tris buffer under constant stirring at 25 °C for six days. The specimens were then rinsed in DI water for 30 minutes 3x and then for 24 hours with constant shaking. They were then remineralized immediately or lyophilized for further characterization.

2.2 Tripolyphosphate (TPP) Pre-Treatment

Following similar protocols to Dai *et al.*, half of the specimens were pre-treated with sodium tripolyphosphate (Fisher) solution prior to remineralization by dissolving 0.106 and 0.245 M TPP in DI water. The pH was adjusted to 7.4 via HCl addition. Just prior to remineralization, the specimens were placed in 300 mL of either low TPP (0.106 M) or high TPP (0.245 M) solution while shaking for 1 hour. Thenspecimens were rinsed in 300 mL of DI water while shaking for 30 minutes immediately prior to placement into mineralization solutions.[8]

2.3 Remineralization

50 mM Tris buffer solution was prepared with 0.9% NaCl and 0.02% sodium azide. The solution was then divided in half and 9 mM $CaCl_2 \bullet 2H_2O$

was added to one half, while 4.2 mM K_2HPO_4 was added to the other half. The solutions were filtered separately using 0.22 µm PES filters and 100 µg/mL 27 kDa pAsp (n=200, Alamanda Polymers) or 2000 µg/mL PAA (1.8 kDa, Sigma) was added to the solution containing $CaCl_2$. The two solutions were mixed together, creating a final mineralization solution of Tris buffer with 4.5 mM calcium, 2.1 mM phosphate, and either 50 µg/mL pAsp or 1000 µg/mL PAA. A group without the addition of pAsp or PAA was also examined. Three samples were added to 500 mL of mineralization solution per group. The solutions were stirred at 150 rpm at 37 °C for two weeks, after which the specimens were rinsed in DI water 3x for 30 minutes while continually shaking. They were then flash-frozen in liquid nitrogen and lyophilized overnight before characterization.

2.4 X-Ray Diffraction (XRD)

Freeze-dried samples were analyzed with a Panalytical XPert Powder by placement atop an amorphous glass slide. Samples were scanned from 10° to 60° (2Θ) with a step size of 0.01° and a dwell time of 10 s/step using Cu Kα x-rays (λ = 1.54 Å).

2.5 Scanning Electron Microscopy (SEM) & Energy Dispersive X-Ray Spectroscopy (EDS)

Freeze-dried samples were mounted onto aluminum stubs with double-sided copper tape. For the interior of the specimens, a razor blade was used to fracture the samples. The mounted samples were sputter-coated with amorphous carbon before analysis with a JEOL 6400 SEM.

2.6 Thermogravimetric Analysis (TGA)

TGA was performed under nitrogen up to 600 °C at a heating rate of 20 °C/min. using a TA Instruments Q5000. Sample masses were ~5 mg.

2.7 Transmission Electron Microscopy (TEM)

Sections of samples were prepared via focused ion beam (FIB) with a Strata-DB235 Dual-Beam (FIB/SEM) instrument. Sections were taken at the surface, 60 µm below the surface, and 100 µm below the surface. To access depths below the surface, the slices were wet sanded with 1200 grit sandpaper and wet sonicated to remove buildup within the tubules. FIBed sections were then analyzed with a JEOL-2010F TEM.

3. Results

Figure 1A shows XRD patterns of native, demineralized, and remineralized dentin. There is little difference between the low and high TPP groups. The groups with XRD patterns most similar to dentin were pAsp, with and without pre-treatment with TPP, with broad HAp peaks indicating nano-sized/impure crystals. The PAA samples contained little to no mineral, as confirmed by SEM micrographs with EDS spectra (data not shown). SEM micrographs of native and demineralized dentin are shown in Figure 1B-D. Figure 1B shows the surface of native dentin. Not many tubules are visible due to mineral occlusion. However, when demineralized, the tubules can be seen (Figure 1D). The interior of native dentin the tubules can be seen , but individual collagen fibrils were not apparent due to the presence of

extrafibrillar mineral (Figure 1C). When demineralized, however, some individual fibrils can be seen in the interior (Figure 1E).

Figure 2A-F shows SEM micrographs of dentin remineralized without polymer. Without TPP treatment, there appears to be large mineral deposits both on the surface and interior, but EDS suggests the collagen is not highly mineralized (Figure 2A&B). With Pre-treatment in either TPP solutions inhibited remineralization on the surface but enhanced it in the interior, judging from morphology (Figure 2C-F). The sharp XRD peaks in these TPP pre-treated groups (Figure 1A) likely indicates the presence of larger, extrafibrillar crystals in the crusty regions (*e.g.*, top of Figure 2F).

When remineralized with pAsp, the surfaces of the slices were covered in a mineral crust, with many tubules occluded, similar in microstructure to native dentin (Figure 2G vs. Figure 1B). The interiors of these samples were similar to native dentin microstructures, but appeared less mineralized, which was confirmed with EDS (Figure 2H vs. Figure 1C). When pre-treated with TPP, the specimens did not exhibit thick mineral crusts on the surfaces (the tubules were not as occluded), but EDS shows the surfaces were highly mineralized (Figure 2I&K). The interiors of these specimens exhibited similar microstructures to native dentin and were more highly mineralized than the specimens without TPP pre-treatment (Figure 2J&L). TGA revealed mineral contents between 51 and 64 wt.%, with TPP pre-treatment enhancing overall remineralization (Table 1). Since 0.245 M TPP pre-treated samples exhibited mineral amounts closest to native dentin (70 wt.%), TEM analysis was

performed on these specimens and compared with specimens remineralized using pAsp alone.

Figure 3A&B shows TEM micrographs of native dentin prepared via FIB. When Remineralization via pAsp with or without 0.245 M TPP yielded surfaces with similar nanostructure to native dentin (Figure 3C&F vs. Figure 3A&B). In both groups, the crystals do not seem preferentially located within gap zones. At 60 µm below the surface, little mineral was present (Figure 3D&G). At 100 µm below the surface, the nanostructures were similar to native dentin, but the crystals appear thicker (Figure 3E&H).

4. Discussion

A high degree of remineralization was achieved using 27 kDa pAsp, with and without pre-treatment with TPP. However, little to no mineralization was detected using 1.8 kDa PAA, even with TPP pre-treatment. Because mineralization solutions containing pAsp precipitated within several days and those containing PAA did not precipitate after 14 days, the likely explanation as to why mineralization did not occur when using PAA is because nucleation was overly inhibited in these systems. Given the low MW of the PAA, it is possible that some of these small polymer molecules entered the collagen fibrils and reduced the Gibbs-Donnan effect, which is presumably what promotes intrafibrillar mineral formation [2, 14]. Likewise, polymer molecules remaining in solution overly inhibited extrafibrillar mineral formation. Tay *et al.* place their collagen substrates on Portland cement blocks during mineralization, which would continuously replenish ions in the mineralization

solution (and it also introduces different ions to the system, such as Si, Bi, Al, F, Fe, and Ti).[15-17] Due to this difference, and that our experiments were performed on bulk specimens instead of individual fibrils like much of Tay *et al.'s* work, the mineralization conditions may need to be further optimized for this polymer type and molecular weight.

TPP treatment of collagen prior to pAsp PILP mineralization enhanced overall mineralization (Table 1). This could be because TPP pre-treatment seemed to inhibit mineralization on the surfaces of the substrates, leaving tubules open through which PILP droplets could penetrate (Figure 2I-L). It is likely that the TPP concentrations at the surfaces of the specimens are greater than in the interiors of the specimens. TPP could be inhibiting mineral formation by chelating Ca^{2+} ions and keeping them from associating with free phosphates. Another reason for the enhancement of mineralization using TPP pre-treatment could be that the TPP molecules have infiltrated the collagen fibrils and associated with the amine groups on collagen, where they may then chelate Ca^{2+} to promote nucleation and growth of HA crystals within the fibrils. Thus, TPP could be acting as the proposed "templating analog", even though enhanced gap-zone banding was not observed. This may lead to higher amounts of mineral than substrates remineralized with PILP alone (Table 1), but apparently only within those regions with the optimal amount of TPP. There were still regions ~60 μm below the surface that showed very little mineral (Figure 3G). This is in accord with our prior studies that find a dip in modulus as it is mapped along the depth of dentin

[13, 18]. This suggests the diffusion limited entry of TPP as well as PILP nanodroplets is creating this non-uniformity of remineralization depth, and alternative methods of delivery may be needed.

5. Conclusions

PAA was unable to effectively remineralize demineralized dentin slices. Although not reported here, simulated body fluid and higher ionic strength solutions were also tested with the addition of PAA. All of these systems failed to produce noticeable mineral in bulk substrates. TPP pre-treatment enhanced the PILP remineralization of demineralized dentin slices. However, neither PILP alone nor PILP with TPP pre-treatment was able to fully remineralize these substrates at all depths over the study period. Although the PILP with TPP pre-treatment did not remineralize the lesions uniformly , this system shows promise in terms of overall mineral content and should be further studied as a possible way to effectively remineralize artificial dentin lesions.

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Figure Legends

Figure 1. XRD patterns of native, demineralized, and remineralized dentin slices (A), and SEM micrographs of native and demineralized dentin slices (B – E). B) SEM micrograph of surface of native dentin, C) interior of native dentin, D) surface of demineralized dentin, and E) interior of demineralized dentin. Scale bars: 10 µm.

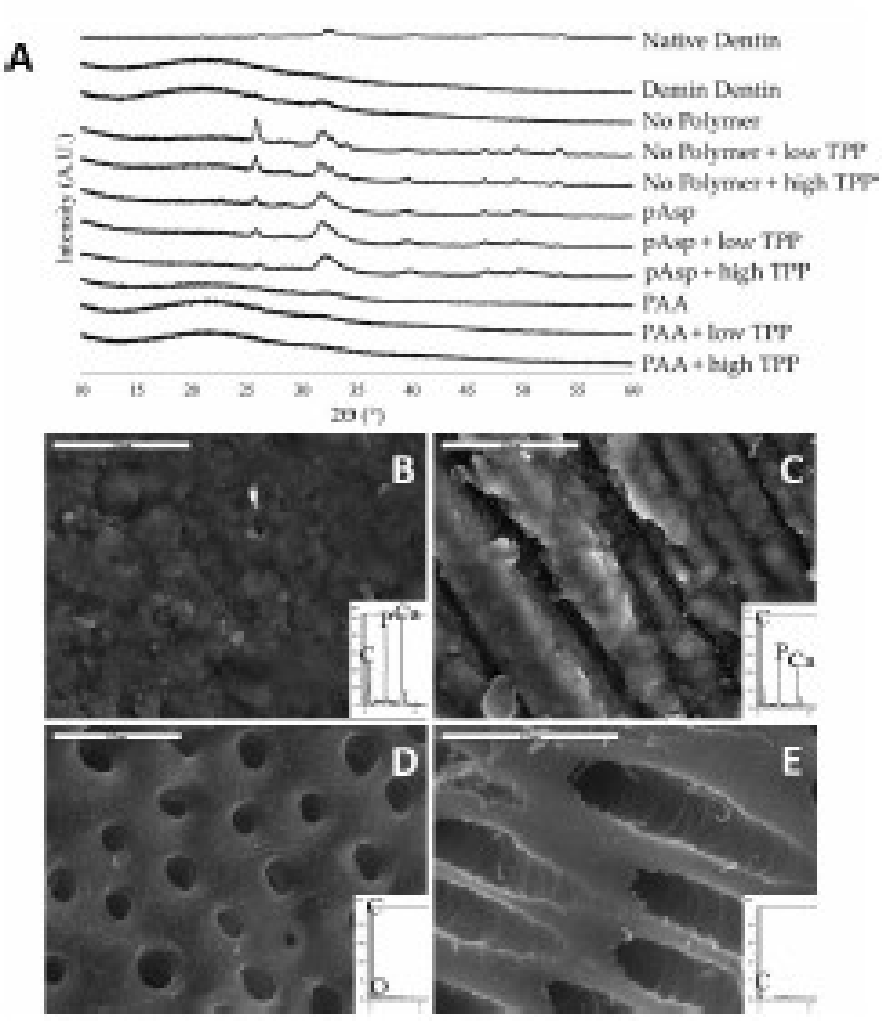


Figure 2. SEM micrographs of dentin slices mineralized in PILP solution and no process-directing agent (A – F), and mineralized in PILP solution with pAsp (G – L). A) surface, polymer and no TPP pre-treatment, B) interior, no polymer and no TPP pre-treatment, C) surface, no polymer and 0.106 M TPP pre-treatment, D) interior, no polymer and 0.106 M TPP pre-treatment, E) surface, no polymer and 0.245 M TPP pre-treatment, and F) interior, no polymer and 0.245 M pre-treatment. G) Surface, pAsp and no TPP pre-treatment, H) interior, pAsp and no TPP pre-treatment, I) surface, pAsp and 0.106 M TPP pre-treatment, J) interior, pAsp and 0.106 M TPP pre-treatment,

K) surface, pAsp and 0.245 M TPP pre-treatment, and L) interior, pAsp and 0.245 M pre-treatment.

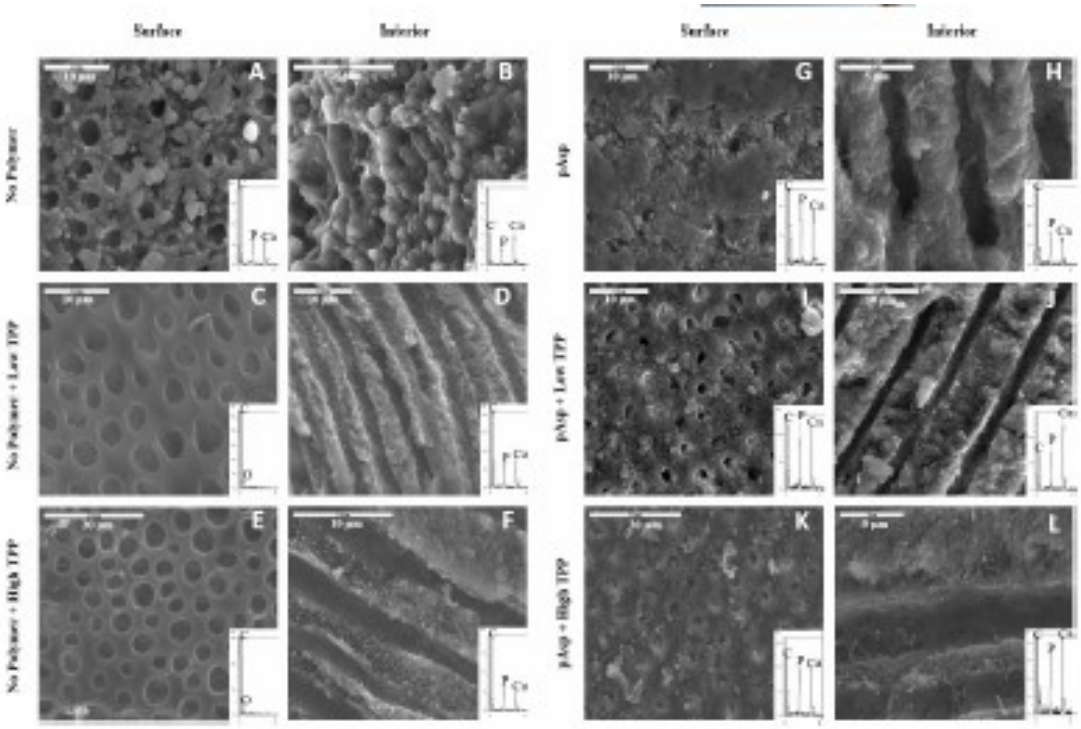
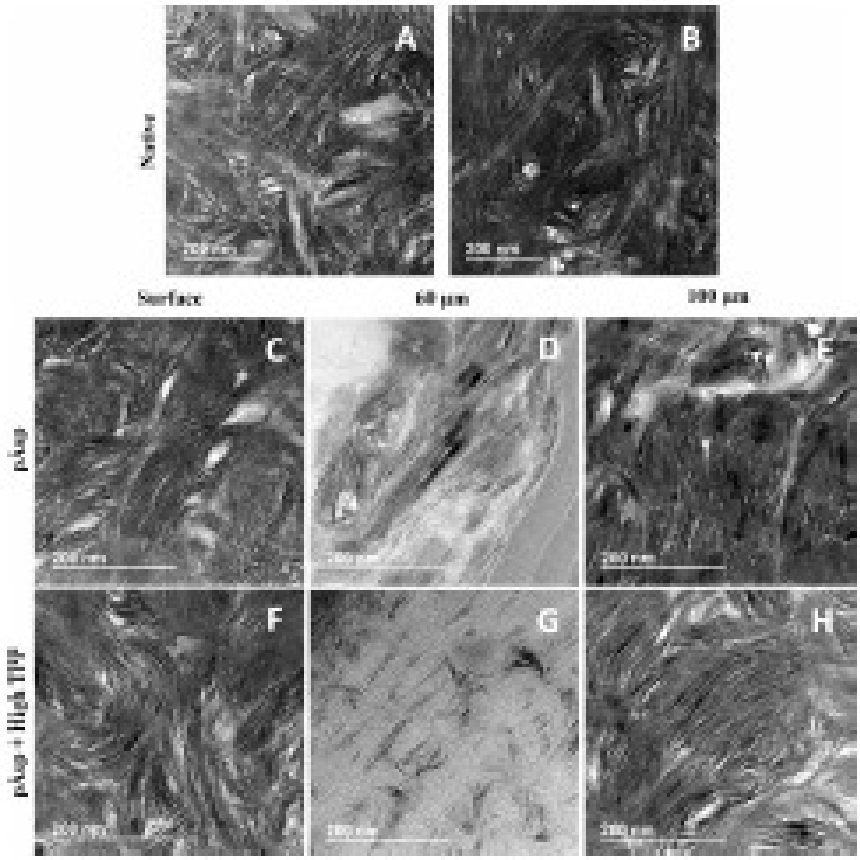


Figure 3. TEM micrographs of native dentin (A & B) and demineralized dentin slices remineralized in PILP solution with pAsp (C – F), with and without 0.245 M TPP pre-treatment. C) Surface, no TPP, D) 60 µm below surface, no TPP, E) 100 µm below surface, no TPP. F) Surface, with TPP, G) 60 µm below surface, with TPP, and H) 100 µm below the surface, with TPP.


Native
A
B
Surface
60 μm
100 μm
p-kup
C
D
E
p-kup + High TPP
F
G
H

Tables

Sample	Mineral Amount
pAsp	51.9 wt.%
pAsp + high TPP	63.4 wt.%
pAsp + low TPP	61.3 wt.%

Table 1. Mineral weight percentages determined by ash weight from thermogravimetric analysis. All values are ± 2.2 wt.%